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Heterodimeric *V. nikolskii* phospholipases A2 induce aggregation of the lipid bilayer

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ABSTRACT

We report that the action of the heterodimeric phospholipases A2 (PLA2s) from *Vipera nikolskii*, which comprises enzymatically active basic subunit and inactive acidic PLA2 homologue, on the lipid bilayer results in the aggregation and stacking of bilayers. These processes are demonstrated using two independent methods (fluorescence spectroscopy and electron microscopy). Aggregation of bilayers is possible because both subunits of the *V. nikolskii* heterodimer contain a membrane-binding site (also known as IBS). Thus, when the two IBSs bind to the membrane, the heterodimer acts as a connecting agent. Heterodimers induce aggregation of negatively charged bilayers composed of phosphatidylglycerol and do not induce aggregation of neutral bilayers composed of phosphatidylcholine.

1. Introduction

Components of venoms receive a lot of researcher attention because they are crucial for the production of antivenoms (Alirolet al, 2010; Isbister, 2010; Theakston et al., 1997; Habib and Warrell, 2013) and possess the potential for the development of new drugs (Chanet al, 2016; Offermanet al, 2002; De Barroset al, 2016). Venoms of different animals from across the globe contain neurotoxins that act on pre- and postsynaptic membranes. Heterodimeric phospholipases A2 are one of such neurotoxins. Phospholipase A2 (3.1.1.4, PLA2) is an enzyme that splits off the acyl chain of a phospholipid at the *sn*-2 position. Heterodimeric PLA2s are composed of two subunits but only one of these subunits has enzymatic activity.

Heterodimeric phospholipases from snake venoms are distinctive[2] in that their subunits A and B are similar in size and are held

Abbreviations: IBS, interfacial binding site; PLA2, phospholipase A2; HDP, heterodimeric phospholipase A2 from *Vipera nikolskii*; V. urs., *Vipera ursinii*; FRET, Forster resonance energy transfer; TMB-PC, 1,3,5,7-tetramethyl-BODIPY-labeled phosphatidylcholine; BCHB-PC, bis-cyclohexyl-BODIPY-labeled phosphatidylcholine; POPC, palmitoyloleoylglycerophosphocholine; POPG, palmitoyloleoylglycerophosphoglycerol; TLC, thin-layer chromatography; TEM, transmission electron microscopy.

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[2] Heterodimeric PLA2s from many venoms of snakes and scorpions have been described. Scorpion neurotoxins (e.g., *Pandinus imperator*, *Heterometrus laoticus*, *Anuroctonus phaiodactylus*, and *Hemiscorpius lepturus*) have two subunits (a small and large one) which are linked by a disulfide bridge (Condeet al, 1999, (Incamnoi et al, 2013), (Valdez-Cruz et al, 2004), (Zamudio et al, 1997))– (Jridiet al, 2015). The large subunit in heterodimers from *Pandinus. imperator* (imperatoxin I and phospholipin) and *Heterometrus. laoticus* (heteromtoxin) scorpions is a phospholipase that is similar to the honeybee phospholipase (Condeet al, 1999). The role of the small subunit is poorly understood, probably it is required to maintains the correct three-dimensional structure of the heterodimer (Zamudioet al, 1997). β-Bungarotoxin from many-banded krait (*Bungarus multicinctus*) is very similar to the heterodimeric scorpion toxins – it is composed of two differently sized subunits linked by a disulfide bridge (Rugolo et al., 1986). Its large subunit has a weak phospholipase activity. Its small subunit is structurally similar to Kunitz-type protease inhibitors although it has no inhibitory activity. This subunit acts as a chaperone that guides the heterodimer to a specific binding site on the synaptic membrane (Banumathiet al, 2001a). Then the binding of the large subunit and the hydrolysis of phospholipids in the membrane increase the membrane permeability to ions and induce the release of neurotransmitters (Rowan, 2001). In addition to heterodimers venoms of some snake species contain non-covalent PLA2 complexes with other or similar proteins. For instance Trimucrotoxin (*Trimeresurus mucrosquamatus*) is a homodimer of crotoxin B like PLA2s. [16)]. Another CB related PLA2 complex – agkistrodotoxin (*Agkistrodon halys Pallas*) could form not only homodimers but "dimer-of-dimers" like structure (Tang et al., 1999). More complicated examples are complexes with non-PLA2 chaperone-proteins like heterotrimeric Taipoxin, oligomeric Taicatoxin (both from *Oxyuranus s. scutellatus*) or heteropentameric Textilotoxin *(Pseudonaja textilis*) (Doley and Kini, 2009).

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together by noncovalent interactions. The subunits are structurally similar; within groups IA and IIA of calcium-dependent snake phospholipases A2, the amino acid sequence identity between subunits A and B is not less than 60%. However, the subunits are inequivalent. Only subunit B has enzymatic activity, whereas only subunit A is highly soluble in aqueous medium. The amino acid sequence of subunit A contains substitutions that impede its binding to the membrane. Moreover, the subunits are charged differently.

One of the first known heterodimers —crotoxin neurotoxin from the venom of *Crotalus durissus terrificus* snake—was isolated in the late 1930s. For this heterodimer, it has been found that the kinetics of the binding between the active subunit and the membrane depends on the presence of subunit A[3] (Faure and Bon, 1988). Other heterodimeric PLA2 with similar structure and catalytic action were later found in different viper species (including some of pit vipers) (Janet al, 2002; Lomonteet al, 2015; Atanasovet al, 2012; Tchorbanovet al, 1977; Tchorbanovet al, 1978).

Most of the studied neurotoxic PLA2 heterodimers from snakes act on the main blood cells and on immunocompetent cells. For instance, crotoxin inhibits the secretion of cytokines and the proliferation of spleen cells (Almeidaet al., 2015), viperotoxin F has a very low anticoagulant activity (Perbandtet al, 2003a), and the heterodimers from *V. nikolskii* venom can markedly reduce platelet aggregation (Gaoet al, 2009).

The venom of *V. nikolskii* viper is distinctive among venoms of other members of the family in that it contains not one but two heterodimeric PLA2s (HDP-1 and HDP-2), the structures of which are typical of snake venom heterodimers (Gaoet al, 2005). Both phospholipases cause blood clotting and reduce platelet aggregation. Each of their subunits fit into the second group of PLA2s. The differences in the kinetics of hydrolysis catalyzed by HDP-1 and HDP-2 are associated with the substitutions of amino acid residues that do not belong to the active site but affect the membrane-binding site. The enzymatic activity of HDP-1 is higher than that of HDP-2 (Ramazanovaet al, 2008).

The mechanism of the toxic action of these heterodimers remains unclear. It is known that binding of their active part (subunit B) with the membrane surface enables heterodimer to dissociate into subunits (Perbandtet al, 2003a; Georgievaet al, 2004). In some cases, the dissociation requires a strongly acidic pH (Komoriet al, 1996). After the dissociation, subunit B can exhibit enhanced phospholipase activity. However, the toxic effect of snake phospholipases is not limited to their phospholipase activity: it was found that some heterodimers from viper venoms contain a specific non-catalytic site that is crucial for the manifestation of toxic effects (Banumathiet al, 2001a; Almeidaet al., 2015). The structure of this site remains unknown.

In many cases, the role of the heterodimeric structure itself and the role of noncatalytic subunit A are unclear. This knowledge gap makes it impossible to understand the mechanism of action of heterodimeric phospholipases.

In the present study, we report that the action of heterodimers from *V. nikolskii* on the lipid bilayer results in the aggregation and stacking of bilayers. These processes damage the bilayer and may cause the toxic effect.

2. Materials and methods

2.1. Materials

Palmitoyloleoylglycerophosphoglycerol (POPG) and palmitoyloleoylglycerophosphocholine (POPC) were obtained from Avanti Polar Lipids; 1,3,5,7-tetramethyl-BODIPY-labeled phosphatidylcholine (TMB-PC) (Boldyrevet al, 2007) and bis-cyclohexyl-BODIPY-labeled phosphatidylcholine (BCHB-PC) (Alekseevaet al, 2016) were synthesized as previously reported.

V.urs.PLA2 from *Vipera urscinii* venom (Tsaiet al, 2011) and v.nik.PLA2 heterodimer phospholipase 1 and v.nik.PLA2 heterodimer phospholipase 2 from *Vipera nikolskii* venom were isolated as described elsewhere (Ramazanovaet al, 2008). A 0.1 M Tris-HCl buffer (pH 8.5) that contained 0.1 M NaCl, and 5 mM $CaCl_2$ was used to prepare a stock solution of the v.urs enzyme. A citric acid–Na_2HPO_4 buffer (pH 3.3) that contained 5 mM $CaCl_2$ was used for v.nik.PLA2. The concentration of v.urs.PLA2 in stock solutions was $6.8 \cdot 10^{-4}$ M and the respective concentration of HDPs from *Vipera nikolskii* venom was 9.3·10-5 M. In the microscopy experiments designed to compare the behavior of *Vipera urscinii* and *Vipera nikolskii* enzymes, the concentration of PLA2 in the samples was 6.8 μM.

2.2. FRET pair of lipid probes

The structures of the BCHB-PC and TMB-PC probes and their absorption and emission spectra are shown in Fig. 1. The donor is TMB (1,3,5,7-tetramethyl-BODIPY-labeled phosphatidylcholine). Its molar extinction coefficient is ~90,000 M^{-1} cm^{-1} and its quantum yield is ~1. The acceptor is BCHB (bis-cyclohexyl-BODIPY-labeled phosphatidylcholine). Its molar extinction coefficient is 70,000 M^{-1} cm^{-1} and its quantum yield is 0.7. The Förster radius for TMB-PC/BCHB-PC pair is 52 Å.

2.3. Preparation of liposomes

A series of liposome samples was prepared using different ratios of negatively charged lipid POPG and neutral lipid POPC. The following POPG:POPC percentage ratios were used: 100:0, 75:25, 50:50, 25:75, and 0:100. Each type of liposomes contained 0.125 mol. % TMB-PC and 0.375 mol. % BCHB-PC. To prevent the inner filter effect, the concentration of TMB-PC was such that the optical density at the absorption maximum of TMB was 0.05. The liposomes were prepared using the lipid film hydration method and extrusion. Briefly, aliquots of a matrix phospholipid and fluorescent probes were co-evaporated from chloroform–methanol mixture (2:1) in a round-bottomed flask on a rotary evaporator and dried at 7 Pa for at least 40 min. The lipid film was hydrated for 2 h at room temperature in Na_2HPO_4–citric acid buffer containing

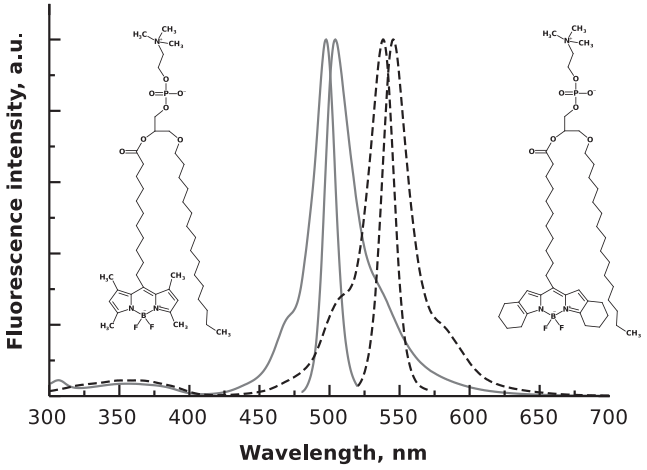

Fig. 1. Excitation and emission fluorescence spectra for TMB-PC (left structure, gray solid lines) and BCHB-PC (right structure, black dotted lines) probes.

[3] Crotoxin subunits A and B are commonly denoted by CA and CB. For unification, we denote them here by A and B in the same way as the subunits of other heterodimers.